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ORIGINAL ARTICLE *Embryology*

Impact of the polycarbonate strippers used in assisted reproduction techniques on embryonic development

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STUDY QUESTION: Do daily manipulations of preimplantation embryos with polycarbonate (PC)—made bisphenol A (BPA)—releasing strippers influence embryo development?

SUMMARY ANSWER: Compared to glass strippers, PC strippers enhance the blastocyst development rate but this does not seem to be BPA-related.

WHAT IS KNOWN ALREADY: PC strippers have been shown to release tiny amounts (around 0.5 ng/ml BPA) of BPA in routine human IVF procedures. A chronic exposure to BPA either *in vivo* or *in vitro* during the preimplantation period can impact post-implantation and post-natal development. BPA can act rapidly by binding to membrane receptors and inducing rapid non-genomic effects.

STUDY DESIGN, SIZE, DURATION: This experimental study using mouse embryos had a balanced design and blinded evaluations of the endpoints.

PARTICIPANTS/MATERIALS, SETTING, METHODS: *In vivo* fertilized zygotes were obtained from outbred Swiss CD1 mice crossings after an ovarian stimulation. The zygotes were allocated to three daily handling conditions (HCs) and cultured until Day 4 in a single human commercial medium. Each day, the embryos were handled for 20 s either in a PC stripper (HC1) or in a glass stripper (HC2). In HC3, the embryos were pre-exposed to 0.5 ng/ml BPA before being handled for 20 s in a glass stripper. Handling operations were repeated on Days 1, 2 and 3. Embryo development was assessed blindly on Day 4. Expanded blastocysts were selected for a transcriptomic analysis using Agilent Sureprint G3 Mouse GE v2 microarrays and the retrotransposon LINE1-*Orf2* expression was analysed using qRT-PCR, as a proxy for a global evaluation of the epigenetic status.

MAIN RESULTS AND THE ROLE OF CHANCE: Compared to the embryos manipulated in HC2 (n = 243), those in HC1 (n = 228) developed significantly more often to the blastocyst stage (55 vs 46%; $P < 0.05$). It appears the effect of these PC strippers was not BPA-related because embryos pre-exposed to BPA (HC3, n = 230) showed no difference in the blastocyst rate when compared to HC2 (43 vs 46%). When analysing same-stage blastocysts, we noticed no difference in the embryo gene expression between the three HC groups.

LARGE SCALE DATA: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148868.

LIMITATIONS, REASONS FOR CAUTION: Our results using a mouse model designed to mimic human conditions (outbred strain, human commercial IVF dishes and a unique commercial human embryonic culture media) are reassuring since no gene was found to be differentially expressed, including LINE-1 genes, as a proxy for a global evaluation of the epigenetic status. However, no global epigenetic

analysis of the genome has been performed. Furthermore, we did not evaluate post-implantation events, although BPA exposure during peri-conception could affect foeto-placental and post-natal development.

WIDER IMPLICATIONS OF THE FINDINGS: Based on the precautionary principle, several European countries banned the use of BPA in baby bottles and food packaging several years before European Agencies took an official position. The question of applying this principle to plastics in closed contact with human embryos is raised. Further studies are needed for a decision to be made.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by a grant from the Agence de Biomédecine (AOR 2016). The authors declare no competing interest.

Key words: bisphenol A / polycarbonate / ARTs / transcriptome / embryonic development

Introduction

Of the environmental toxins that can affect the human reproductive function, bisphenol A (BPA) has been one of the most studied. A review concluded that BPA is reprotoxic in humans, mainly acting on the ovaries and the uterus (Peretz *et al.*, 2014). Several studies carried out in women using ARTs have shown an inverse relationship between the urinary BPA concentration and the antral follicle count (Souter *et al.*, 2013), the number of total collected oocytes (Mok-Lin *et al.*, 2010) and recovered mature oocytes, the oestradiol peak level at the end of ovarian stimulation (Mok-Lin *et al.*, 2010; Ehrlich *et al.*, 2012b), the fertilization rate (Ehrlich *et al.*, 2012b) and, finally, the risk of embryo implantation failure (Ehrlich *et al.*, 2012a). Numerous *in vivo* and *in vitro* studies in animal models have determined that BPA alters the quality of oocyte meiosis (Peretz *et al.*, 2014).

Female mice exposed to high doses of BPA (40 and 100 mg/kg/day) from 0.5 to 3.5 days post-coitum (dpc) (i.e. the preimplantation period of embryo development) showed a decrease in (40 mg/kg/day) or an absence of (100 mg/kg/day) implantation sites at 4.5 dpc (Xiao *et al.*, 2011). The transit of embryos in the fallopian tubes and embryonic development are delayed at 3.5 dpc, with the majority of embryos being found in the fallopian tubes and few have reaching the blastocyst stage (Xiao *et al.*, 2011). Similarly, *in vitro* embryonic development in the presence of a high concentration (100 µM) of BPA is delayed (Takai *et al.*, 2001). Recently, it has been shown that pre- and peri-conceptional exposure to BPA in female mice lead to an altered expression in the foetus at 9.5 dpc of some imprinted genes associated with DNA methylation abnormalities (Susiarjo *et al.*, 2013). This confirmed other studies in which it was demonstrated that BPA can alter the methylation level of parental imprinted genes at the oocyte level via a disruption of oestrogen receptor signalling pathways (Chao *et al.*, 2012). However, in all these studies, the exposure of the oocyte/embryo to BPA was prolonged.

In ART, human oocytes and embryos are submitted to non-physiological handling and culture conditions, especially a particular and intensive use of plastic consumables. We have previously shown that in routine ART conditions, some of these plastic products (stripper tips used for oocyte and zygote denudation and fine embryo manipulation) could release BPA (Gatimel *et al.*, 2016) at concentrations in the ng/ml range, similar to those found in serum and human follicular fluids (Vandenberg *et al.*, 2010). The plastic used for the manufacture of these strippers is polycarbonate (PC), a very well-known BPA polymer than can release BPA monomers. For this reason, BPA-based PC plastics have been banned in the manufacturing of baby bottles in many countries. The handling of human oocytes and preimplantation embryos with PC strippers means they are in close contact with BPA for a few seconds each time they are handled. The consequences of these short contacts remain unknown but must be studied for two reasons. First, apart from the typical nuclear oestrogen receptors, BPA can be linked to various other types of receptors (MacKay and Abizaid, 2018) including membrane receptors such as the G-protein-couple oestrogen receptor (GPER). GPER can regulate rapid cellular responses through the activation of adenyl cyclase and the recruitment of second messenger cascades like cAMP which activates the protein kinase A pathway. GPER can also induce rapid non-genomic effects by mobilizing intracellular calcium following the activation of the PI3K signalling pathways. In mice, GPER is expressed in the oocyte/embryo (Li *et al.*, 2013; Yu *et al.*, 2015) and mediates the fast action of E2 on the blastocyst, inducing intracellular calcium release, and on the cytoskeletal rearrangements (Zhang *et al.*, 2017), affecting its implantation potential (Yu *et al.*, 2015). In humans, GPER protein was detected in the oocyte (Heublein *et al.*, 2012) but has not yet been studied in the embryo, as far as we know. Secondly, the preimplantation period is a time of major expression modifications through epigenetic changes that are sensitive to environmental challenges (Xavier *et al.*, 2019). Under *in vitro* conditions, unlike *in vivo* conditions, this sensitivity is probably increased due to the lack of detoxification mechanisms inherent to the tissue/cell structures in the environment of an oocyte (cumulus and granulosa cells) or embryo (epithelial tubal and endometrial cells). The deleterious effect of culturing mice embryos in the presence of BPA was indeed substantially prevented when the culture was performed on endometrial epithelial cells (Lee *et al.*, 2012).

Therefore, the aim of the study is to assess whether handling embryos with BPA-containing strippers has an influence on embryo development, the transcriptome profile or the epigenetic status in the mouse model.

Materials and methods

Outbred Swiss-CD1 mice (7 weeks) from JANVIER LABS (Le Genest St Isle, France) were housed under the standard European animal care guidelines in a pathogen-free barrier facility (i.e. 22°C ± 1°C, around 50% humidity and 12 h light/dark cycle with a normal chow diet). The facility has been authorized for animal research (agreement n°A31 555 011) since 10/12/2015. Ethical approval was unnecessary for this experiment in accordance with Directive 2010/63/EU. The mice were super-ovulated with a bolus of PMSG (10UI via intraperitoneal route, from Centravet, Lapalice, France). Ovulation was induced by a bolus of hCG (5 UI via intraperitoneal route, from Centravet, Lapalice, France). They were then crossed with males from the same strain.

Mating was verified by the presence of a vaginal plug. Mice were euthanized by cervical dislocation. The zygotes were recovered approximately 20 h after hCG injection by flushing the oviducts with a 37°C M2 media (Sigma Aldrich, Saint Quentin, France) in polystyrene petri dishes (VWR International, USA, ref.: 391-0108). The zygotes were then transferred into dishes with hyaluronidase (Sigma Aldrich, Saint Quentin, France), dissected under stereomicroscope and subjected to three washes with M2 Medium. Finally, isolated one-cell embryos were stored in a 25 µl drop of Global® Total® medium (CooperSurgical, Versailles, France) covered by 7 ml mineral oil (FertiCult, Fertipro, La Mulatière, France) pending further handling.

Handling of zygotes and embryos

Zygotes were divided into three groups corresponding to three different handling conditions (HCs) for 3 days (Fig. 1). Between the daily handling operations, the zygotes and embryos were all cultured in 60 mm diameter IVF-certified polystyrene dishes (VWR International, USA, ref.: 391-0108) in a 25 µl drop of Global® Total® medium overlaid with 7 ml mineral oil (FertiCult, Fertipro, La Mulatière, France) at 37°C, 5% CO_2 in the same incubator. Dishes, media, plastic pipette tips had been previously shown not to release detectable BPA (Gatimel *et al.*, 2016).

As described in Fig. 1, HC1 was a condition mimicking the routine embryo handling operation applied in human ART labs. HC2 was a negative control condition in which embryos are not supposed to interact with BPA. HC3 was a positive control condition in which embryos encountered BPA at 0.5 ng/ml (i.e. in the range of concentration levels previously measured in several brands of PC strippers rinses) (Gatimel *et al.*, 2016). In HC1, the zygotes or embryos were first handled with an IVF-certified glass stripper (Manipulation SG Pipette, SYNGA, Praha, Czech Republic) in 3 µl of medium in order to place them into a 25 µl drop of Global® Total® medium supplemented with 0.33% of dimethylsulfoxide (DMSO) (Sigma Aldrich, Saint Quentin, France) for 15 min. Three microliters of the medium then aspirated into a PC stripper (Denudation pipette, EZ-Tips®, Research Instrument, Versailles, France) for 20 s before being placed into a 25 µl drop of Global® Total® medium for culture until the next day. DMSO was added because in the last condition (HC3), BPA was dissolved beforehand in this vehicle. In HC2, the zygotes or embryos were handled exactly in the same way except for the fact that the stripper maintaining the embryos for 20 s was a glass stripper (Manipulation SG Pipette, SYNGA, Praha, Czech Republic). In HC3, the zygotes or embryos were handled in the same way as in HC2, except for the fact that the 25 µl drop of Global® Total® medium was also supplemented with BPA (Sigma Aldrich, Saint Quentin, France) at 0.5 ng/ml (concentration verified using LC/MS-MS dosage) for a 15 min contact.

Embryo handling was performed 24, 48 and 72 h after hCG injection in the three HC groups. At 96 h, blastocysts were allocated and dry-frozen in RNAse-free tubes for the planned analysis (transcriptomic and retrotransposon analysis).

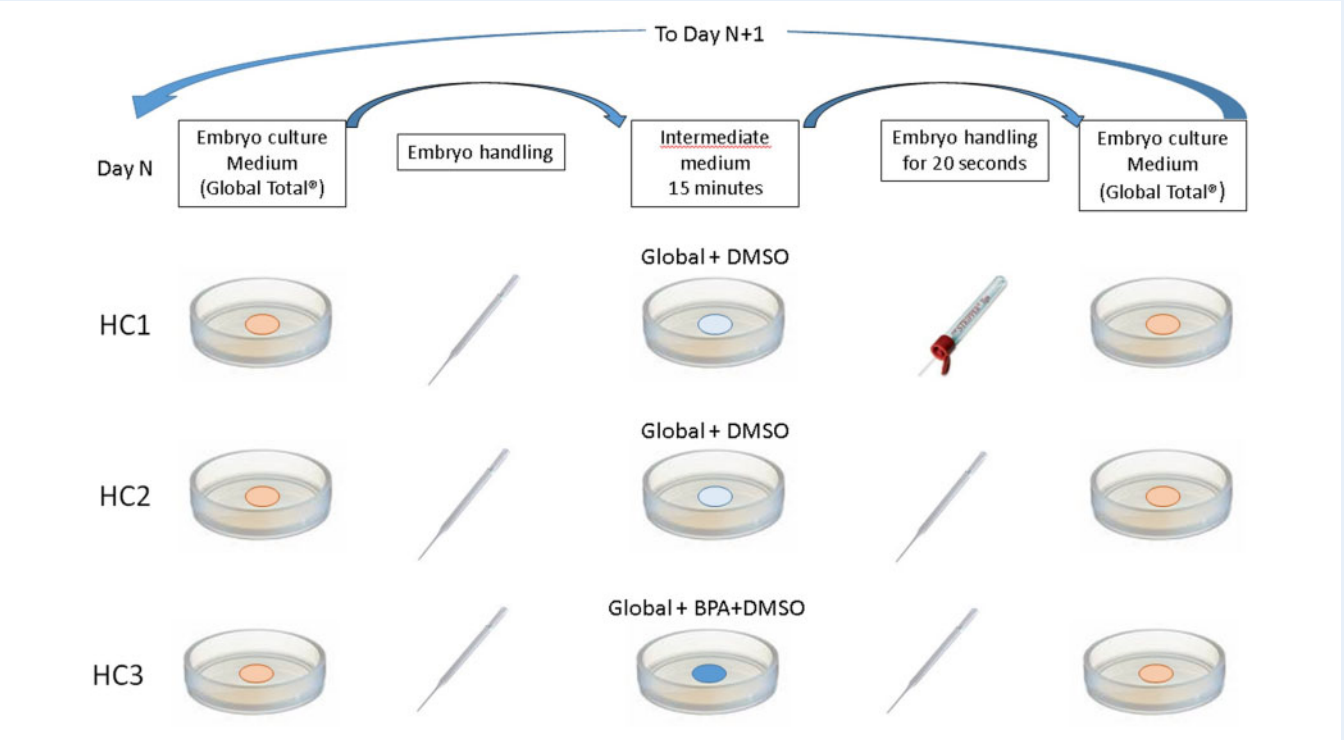

Figure 1. The experimental protocol. Three handling conditions (HCs) of embryos were compared. From the drop of embryo culture medium, the embryos were transferred (by groups of 10) for 15 min in the same medium supplemented with 0.5 ng/ml BPA (HC3) or its vehicle (HC1 and HC2). The embryos were then maintained for 20 s in a glass stripper (HC2 and HC3) or in a polycarbonate stripper (HC1) before being placed back into the culture medium for an additional 24 h culture. This was done at 24, 48 and 72 h post-coïtum.

Measurements of BPA concentrations

The HC3 condition was selected based on our previous results on three brands of strippers (Gatimel et al., 2016). In order to check if the strippers used in 2020 (from a unique manufacturer) gave the same results, we measured the concentrations of BPA released by those strippers in routine-like conditions using the same methodology as previously described (Gatimel et al., 2016) excepted that each stripper was filled with 3 µl of Global® Total® medium. Three different batches of strippers were tested, each in triplicate. Briefly, for one replicate, 20 strippers from the same batch were pre-warmed at 37°C and individually filled with 3 µl taken from a 25 µl drop of 37°C warmed Global® Total® medium. After 20 s of contact, each 3 µl were added to the same Eppendorf 1.5 ml tube for final storing at −20°C. We also re-tested the embryo culture medium (two batches in triplicate) and the oil (one batch in triplicate) by taking 50 µl directly from their commercial vials. Finally, two microdrops of 30 µl of the embryo culture medium were incubated at 37°C, 5% CO_2 for 24 h under oil in the same dish as those used for culturing the mouse embryos, then 25 µl from each drop was taken and pooled in an Eppendorf 1.5 ml tube. This was done in triplicate for two batches of the medium. The plastic tips and tubes used to sample and store the samples had been previously excluded as they did not release detectable BPA (Gatimel et al., 2016).

Evaluation of embryo development

For each HC group, embryo development was assessed each day by the same embryologist (J.M.) at the time of embryo handling. At Day 2, the number of eight cells embryos was noted and at Day 3, the number of compacted morula was noted. On Day 4, 91 embryos were photographed and assigned a three-digit random number using the Xcel software. The pictures were ranked by ascending order of the random number and blindly given to a second embryologist (R.D.L.). The number as well as the type of blastocysts obtained (young i.e. an embryo with a blastocoel cavity whose diameter has not increased, expanded or hatched) were assessed. Percentages were calculated from the number of two cells embryos assigned in each condition. Variables were expressed as numbers (percentages) and compared using the χ^2 or Fisher's exact test as appropriate.

RNA extraction for the transcriptomic analysis

RNA extraction was performed on pools of 15 frozen blastocysts and 4 pools per HC group. As the embryo gene expression is dynamic and highly dependent on the embryo stage, we chose to compare gene expression between the three HC groups on the expanded blastocysts only. We used the Arcturus PicoPure™ RNA isolation kit (LifeTechnologies, Courtaboeuf, France) for the RNA extraction. Briefly, 100 µl of extraction buffer was added to each pool of embryos. The samples were then incubated at 42°C for 30 min and then 100 µl of ethanol (70%) was added to each sample. The samples were then deposited on columns previously incubated with 250 µl of conditioning buffer. After two centrifugations, the columns were washed once with 100 µl of washing buffer. The samples were then incubated with 40 µl of DNAse (Qiagen, Courtaboeuf, France) for 15 min at 25°C. The samples were then washed three times, and finally, 11 µl of elution buffer was added to the samples. After incubation (1 min) and two centrifugations, the RNA of each sample was isolated and frozen at −80°C until analysis. RNA quantities and qualities were checked using the Agilent 5200 Fragment Analyzer System. The mean total RNA concentration was 1.44 ± 0.46 ng/µl and RNA quality was good (RNA quality numbers and 28S/18S ratios respectively as means ± SD: 9.37 ± 1.36 and 2.38 ± 0.77). A one-way ANOVA was performed to compare the RNA concentrations between HC groups.

Transcriptome analysis

Microarray gene expression profiles were performed at the GeT-TRiX facility (GénoToul, Génopole, Toulouse, Midi-Pyrénées) using Agilent Sureprint G3 Mouse GE v2 microarrays (8x60K, design 074809) following the manufacturer's instructions. For each sample, Cyanine-3 (Cy3) labelled cRNA was prepared from 2 ng of total RNA using the One-Color Quick Amp Labelling kit (Agilent Technologies) in accordance with the manufacturer's instructions, followed by Agencourt RNAClean XP (Agencourt Bioscience Corporation, Beverly, Massachusetts). Dye incorporation and cRNA yield were checked using Dropsense™ 96 UV/VIS droplet reader (Trinean, Belgium). Then 600 ng of Cy3-labelled cRNA was hybridized on the microarray slides in accordance with the manufacturer's instructions. Immediately after washing, the slides were scanned on an Agilent G2505C Microarray Scanner using Agilent Scan Control A.8.5.1 software and the fluorescence signals were extracted using Agilent Feature Extraction software v10.10.1.1 with default parameters.

Microarray data and experimental details are available in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE148868. Statistical analysis of the microarray data was performed using R (R Core Team, 2018) and Bioconductor packages (Huber et al., 2015) as described in GEO accession GSE148868. The raw data (median signal intensities) were filtered, log2 transformed and normalized using the quantile method (Bolstad et al., 2003). A model was fitted using the limma lmFit function (Ritchie et al., 2015). Pair-wise comparisons between biological conditions were applied using specific contrasts. A correction for multiple testing was applied using the Benjamini–Hochberg (BH) procedure (Benjamini and Hochberg, 1995) to control the False Discovery Rate (FDR). Probes with FDR $\leq$0.05 were considered to be differentially expressed between the conditions.

LINE-1 (*Orf2*) analysis

LINE-1 is a transposable element (TE) which represents 17% of the human genome. It has two open reading frames that encode the proteins required for its retrotransposition. Although the vast majority of LINE-1 elements have been inactivated during evolution, i.e. they are unable to retrotranspose, a few of them (about 80–100) are still active and have been widely studied in many health subject matters such as oncology, embryology, neurology and psychiatry (Del Re and Giorgi, 2020). Methylation of DNA at CpG dinucleotides of LINE-1 promotor restricts its expression during preimplantation development (Yandım and Karakülah, 2019). Recently, LINE-1 (*Orf2*) expression in mice blastocysts has been shown to be sensitive to *in vitro* culture and media composition (Carmignac et al., 2019) and appeared to be decreased in cord blood from ART new-borns compared to naturally conceived ones (Choux et al., 2018). Therefore, evaluation of LINE-1 (*Orf2*)

expression by embryos is used as a proxy for an epigenetic impact of the HC.

Sample preparation

The embryos were frozen in pools of five blastocysts. Then the extraction of the RNA was carried out on each of the pools blastocysts using the Arcturus PicoPure™ RNA isolation kit (LifeTechnologies, Courtaboeuf, France) with an additional DNase digestion step (TURBO DNA-free™, LifeTechnologies, Courtaboeuf, France). Reverse transcription was realized using SuperScript™ III Reverse Transcriptase (LifeTechnologies, Courtaboeuf, France). At least three pools of blastocysts per condition were included for analyses.

LINE-1 (*Orf2*) analysis

A quantitative PCR (qPCR) was performed in order to study the expression of the LINE-1-transcript (*Orf2*), a TE using the Maxima SYBR Green/ROX qPCR Master Mix (LifeTechnologies, Courtaboeuf, France) as previously described (Carmignac *et al.*, 2019). The level of expression of LINE 1 (*Orf2*) was normalized to the geometric mean of the expression levels of 2 housekeeping genes (*Gapdh*, *Ppia*) according to the formula: LINE 1 (*Orf2*)/geometric mean (*Gapdh*, *Ppia*) $=2^{(Ct[LINE\text{-}1\ (Orf2)]\text{-arithmetic mean }[Ct(Gapdh),Ct(Ppia)])}$, where Ct is the threshold cycle. Condition HC2 was taken as a reference for the study of LINE-1 (*Orf2*) expression in HC1 and HC3 using the $2^{-\Delta\Delta\ Ct}$ calculation. Data were analysed with Bio-Rad CFX Manager (Version 3.0.1224.1015). The primers used were 5′TCCATGACAACTTTGGCATTG3′ (F) and 5′CAGTCTTCTGGGTGGCAGTGA3′ (R) for *Gapdh*, 5′CGCGTCTCCTTCGAGCTGTTTG3′ (F) and 5′TGTAAAGTCACCACCCTGGCACAT3′ (R) for *Ppia* and 5′GGAGGGACATTTCATTCTCATCA3′ (F) and 5′GCTGCTCTTGTATTTGGAGCATAGA3′ (R) for LINE-1.

Statistical analysis

Percentages were compared using the χ^2 test. Continuous variables were expressed as means ± SDs, and compared using the Student's or Mann–Whitney test, as appropriate. Statistical analysis was performed with SAS software, v. 9.4 (SAS Institute Inc, USA). A *P*-value <0.05 was considered significant.

Results

BPA measurements

BPA measurements are presented in Supplementary Table SI. The culture medium and the oil did not contain quantifiable levels of BPA. The same was true after incubating microdrops of medium for 24 h on the IVF dishes in the conditions used to culture mouse embryos. Finally, all three batches of strippers released BPA in the medium contained in their interior volume to a mean concentration of 1.1 ± 0.2 ng/ml.

Embryonic development

After recovery, 262, 293 and 262 zygotes were respectively allocated to the HC1, HC2 and HC3 groups. Embryo development at Day 2 and Day 3 was similar between the three conditions (Table I). Handling embryos with plastic strippers (HC1) yielded an increased blastocyst rate compared to glass strippers (HC2) (55 vs 46%; $P<0.05$) and to a short exposure to BPA (HC3) (55 vs 43%; $P<0.05$). However, in the latter experimental condition, blastocysts were significantly less expanded compared to those in HC1 and HC2 (Table I).

Table I Evaluation of embryo development in the three experimental groups.

Handling conditions	HC1	HC2	HC3
Day 1. 2-cell embryos	228	243	230
Day 2. 8-cell embryos (% 2 cell embryos)	216 (95)	232 (95)	213 (93)
Day 3. Morulas (% 2 cell embryos)	198 (87)	200 (82)	192 (83)
Day 4. Blastocysts (% 2 cell embryos)	126 (55)	112 (46)*	100 (43)*
Expanded blastocysts (% of blastocysts)	59 (47)	61 (54)	31 (31)*,†
Expanded or Hatched blastocysts (% of blastocysts)	77 (61)	74 (66)	49 (49)†

*$P<0.05$: significant difference compared with HC1.
†$P<0.05$: significant difference compared with HC2.

Transcriptomic analysis

Prior to performing the transcriptomic analysis, we evaluated a possible difference in RNA amounts between the HC groups using a one-way ANOVA. The result indicates that there was no significant difference ($P=0.812$) in RNA concentrations according the HC groups, and thus no impact can be expected on the generation of transcriptomes. All the samples exceeded the minimum labelling yield and specific activity values recommended by Agilent (0.825 µg cRNA and 6 pmol Cy3/µg cRNA). The background noise of the all microarray was homogeneous and below (mean intensity of 25) the value recommended by Agilent (intensity < 40). A Principal Component Analysis was performed on normalized expression data to illustrate the main sources of variability. The first two components, gathering 37% of the gene expression variability between all samples, failed to show evidence of a clear influence of the HC groups on the transcriptome (Fig. 2). Differential gene expression analysis did not identify any statistical difference between conditions HC1, HC2 or HC3 after correction for multiple testing using the BH procedure (adjusted *P*-value < 0.05).

LINE-1 (*Orf2*) analysis

Taking HC2 as reference, the level of expression of LINE-1 (*Orf2*) was not statistically different in the three different conditions ($P<0.05$) (Fig. 3).

Discussion

We previously showed that human embryos could be in close contact with weak concentrations (around 0.5 ng/ml i.e. 2.19 nM) of BPA inside PC strippers from three different manufacturers (Gatimel *et al.*, 2016),

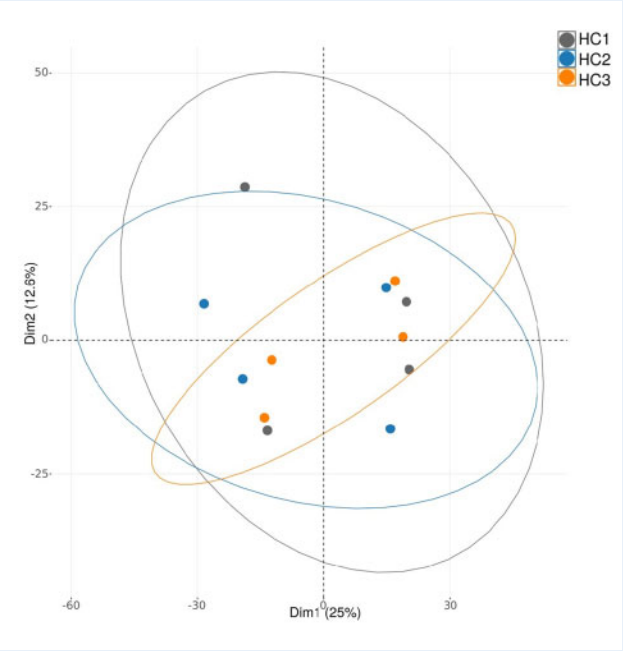

Figure 2. Principal component analysis of transcriptomic data. Normalized expression signals were subjected to a principal component analysis. The four replicates in each condition (HC1, HC2 and HC3) were projected to components 1 and 2. Ellipses illustrate 95% confidence region for each condition.

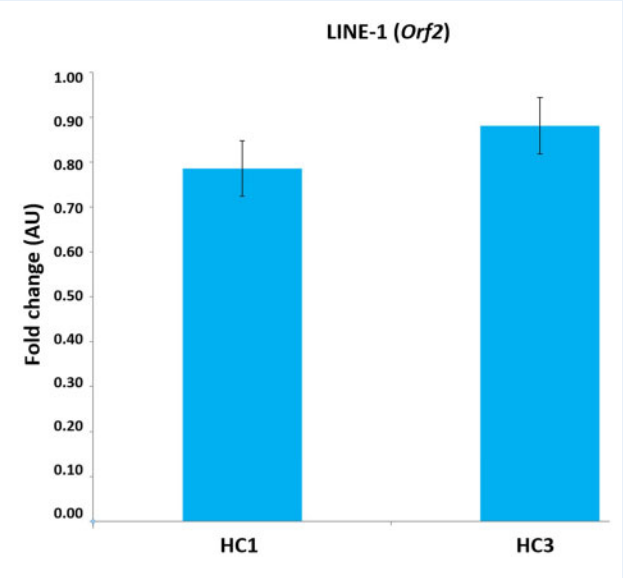

Figure 3. Relative expression of LINE-1 (*Orf2*) under conditions HC1 and HC3 as measured by RT-qPCR. The data are normalized to *Gapdh and Ppia* and expressed as the fold change pertaining to control group HC2 (condition HC2 was taken as a reference: HC2 fold change = 1). Data represent mean ± SEM from at least three biological replicates (five blastocysts per replicate).

which prompted us to assess the embryonic effects of such an exposure.

Interestingly, the handling of embryos in PC strippers yielded better development to the blastocyst stage compared to glass strippers (HC1 vs HC2) or to low (0.5 ng/ml or 2.19 nM) and short daily (15 min) exposures to BPA (HC1 vs HC3). We have no argument to consider that handling embryos with glass strippers could have a toxic effect on embryo development. Firstly, we used IVF-certified and MEA-tested glass strippers. Secondly, two studies compared mice embryo culture from the zygote stage into glass capillaries (10 embryos in 25 µl of culture medium (Popova *et al.*, 2011)) or microcapillaries (2 embryos in 1 µl of culture medium (Thouas *et al.*, 2003)) to control cultures in plastic dishes. Blastocyst rates were either increased (Popova *et al.*, 2011) or identical but with larger proportions of partially or fully hatched blastocysts (Thouas *et al.*, 2003) using glass materials.

At the same time, our results show that a short (15 min) daily embryonic exposure to low concentrations of BPA had no influence on development to the blastocyst stage as HC2 and HC3 yield the same blastocyst rates. So far, the three studies assessing the impact of BPA exposure on *in vitro* preimplantation development in mice have used a chronic exposure covering the entire culture period. These studies constantly revealed a decrease in blastocyst rates after exposure to high concentrations (100 µM) of BPA (Takai *et al.*, 2000, 2001; Lee *et al.*, 2012). In contrast, in these same studies, lower doses of BPA exposure resulted either (1 nM and 3 nM) in a slight increase in blastocyst rates (Takai *et al.*, 2000, 2001) or in non-significant change (10 nM, Lee *et al.*, 2012). Apart from mice, one study reported a lower blastocyst rate after the exposure of eight-cell bovine embryos to BPA at 43.8 nM (10 ng/ml) for 96 h and no differences at a concentration of 4.38 nM (1 ng/ml) (Choi *et al.*, 2016). Brought together, the observations on blastocysts rates in our study argue in favour of the conclusion that the plastic strippers have positive effects on embryo development but that they are not BPA-related. PC manufacturing processes not only use BPA but also several other plastic additives affecting the product's final aspect, transparency and flexibility. One can speculate that some of these additives could be responsible for the observed effects. This is a difficult hypothesis that requires further studies. The questions would be more easily answered if manufacturers were involved and gave access to accurate data. Our positive control HC3 contained 0.5 ng/ml BPA based on our previous results on three different brands of plastic strippers (Gatimel *et al.*, 2016). Here we checked the release of BPA for one of these brands and observed, using exactly the same methodology as in 2016, a higher BPA release (1.1 ng/ml). In our opinion, this higher released could not explain the higher blastocyst rate observed in HC1 versus HC3 since it is known that the non-monotonic relationships between BPA concentration exposure and *in vitro* embryo development in the mouse required at least a factor of 10 difference in BPA concentrations to be observed (Takai *et al.*, 2000; Lee *et al.*, 2012). We do not know if the manufacturer performed any change in the manufacturing process between 2016 and 2020. As far as we know, only one manufacturer claims to propose, since 2018, a stripper made out from polyamide that is 'considered to be bisphenol A (BPA) free' as mentioned on their website.

Different results have been found concerning blastocyst morphology. In our study, blastocyst expansion was delayed only after BPA exposure as the proportion of expanded blastocysts as well as expanded or hatched blastocysts was lower in HC3 compared to the other

groups. These results are at odds with those of Takai *et al.* (2000, 2001) that did not find a morphological difference between embryos exposed to BPA and non-exposed embryos. Differences in doses, modalities of BPA exposure, use of an outbred strain of mice and the blind analysis of embryo morphology render the comparison of these results difficult. In the bovine model, no differences were found in the type of blastocysts (expanded or not) regardless of the level of BPA exposure (1 ng/ml or 10 ng/ml, Choi *et al.*, 2016).

In conditions designed to mimic the human situation (outbred strain of mice, 20 s exposure to the stripper once a day for 3 days, plastic labware and culture medium used for human IVF), our results are reassuring. After comparing same-stage mice blastocysts obtained after being handled either in plastic strippers or in glass strippers or after a short exposure once a day to 0.5 ng/ml BPA, we did not identify difference in their gene expression nor in their expression for the retrotransposon LINE-1 (*Orf2*). Because the embryo transcriptome is highly dynamic during the preimplantation development, we want to stress out the tremendous importance of comparing same-stage embryos when assessing the effects of an external factor on the embryo gene expression. Otherwise, should this factor delay the embryo development for example, one could conclude by mistake that it also affects the gene expression while the differential gene expression only reflects the differential development progress. To our knowledge, this is the first data on the impact of BPA on global gene transcription during mammalian preimplantation embryonic development. Using bovine embryos, Choi *et al.* (2016) demonstrated differences in the blastocyst development rate after exposure to 10 ng/ml, but no difference on blastocyst morphology or on the expression of the panel of genes analysed by RT-qPCR. Although there is no data showing the consequences of gene deregulation after *in vitro* exposure to BPA on preimplantation embryonic development, some data are available on post-implantation embryonic development. Susiarjo *et al.* (2013) observed a change in the genes subjected to parental imprinting in embryos and placentas at the embryonic days 9.5 and 12.5 when the mother had been exposed to 10 mg/kg bw/day of BPA in the periconceptional period. Albeit the absence of gene expression deregulation, we have shown that a single daily embryo handling with PC strippers can affect the preimplantation embryo's developmental abilities, therefore it would be interesting to check if it also influences long-term development. After the *in utero* transfer of *in vitro* cultured embryos in the presence of BPA, Takai *et al.* (2001) did not show any differences in immediate postnatal data (number of pups per litter, birth weight and sex ratio) between the groups exposed or not to BPA. However, at weaning (postnatal day 21) they showed an sharp increase in the weight of females (+39%) when exposed to BPA, including in low concentrations (1 nM) (Takai *et al.*, 2001). If true, the mechanisms underlying these observations are unknown but probably involve epigenetic programming of the embryo. Using LINE-1 (*Orf2*) expression as a proxy for the embryo epigenetic state, we failed to evidence any impact of short BPA exposures or PC stripper handling operations. However, the limit of our study lies in the fact that we did not analyse the whole epigenome of the embryos.

Conclusion

The process of BPA substitution has already started in other areas, particularly in the food sector for which numerous legislative decisions have prohibited the use of BPA in plastics in contact with food or even early childhood products (Santé Publique France, 2019). However, substitute substances, including bisphenols S and F must also be evaluated given the doubts about their safety (Rochester and Bolden, 2015). Regarding the use of PC strippers in ART laboratories, the data obtained in our mouse embryo model were encouraging, assuming that murine-related data translates to preimplantation humans embryo physiology (Sharpe, 2018). Yet, a blind appliance of the precautionary principle without sufficient arguments may be counterproductive, as our study provides the first clues to help make decisions. Additionally, substituting this material for glass material could be a safer alternative although glass represents a risk of embryo loss due to glass breakage.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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Authors' roles

J.M. carried out all the experiments, participated in their analysis and wrote the article. R.D.L. designed the study, participated in the data analysis and the manuscript writing. G.T. performed mouse superovulations and helped perform the embryo culture. Y.L. and C.N. participated in the transcriptomics study and the analysis of results. P.F. and M.G. participated in the retrotransposons study and the analysis of results. A.A.H. performed the BPA measurements. N.G., V.G. and J.P. participated in the development of the study protocol and proofread this article.

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Conflict of interest

The authors declare that they have no conflict of interest.

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